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Invited Review

Monitoring antimalarial drug resistance: Applying lessons learned from the past in a fast-moving present

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ABSTRACT

The need for robust surveillance of antimalarial drugs is more urgent than it has ever been. In the western region of Cambodia, artemisinin resistance has emerged in *Plasmodium falciparum* and threatens to undermine the efficacy of highly effective artemisinin combination therapies. Although some manfestations of artemisinin tolerance are unique to this class of drug, many of its properties mirror previous experience in understanding and tracking resistance to other antimalarials. In this review we outline the spectrum of approaches that were developed to understand the evolution and spread of antifolate resistance, highlighting the importance of integrating information from different methodologies towards a better understanding of the underlying biologic processes. We consider how to apply our experience in investigating and attempting to contain antifolate resistance to inform our prospective assessment of novel antimalarial resistance patterns and their subsequent spread.

Contents

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1. Introduction

Antimicrobial drug resistance is one of the greatest challenges confronting the control of clinical infectious diseases, affecting viruses, bacteria, fungi and parasites alike. Our antimicrobial armamentarium is often limited and as a result, pathogens respond to the selective pressure of intensive drug use. Under these circumstances, the genetic changes that underlie resistance have a high probability of occurring, and when the pathogen encounters the drug, strains with those genetic changes have a much greater chance of surviving and being transmitted to a new host. When these conditions are met, emergence of pathogens resistant to that drug is inevitable. The public health question is not if, but how quickly resistance will evolve and under what conditions those resistant pathogens will be selected and spread.

The consequences of resistance to antibacterial and antiviral drugs have been widely publicized. The principles, approaches and lessons learned can be applied to any of these pathogens, from Methicillin-resistant *Staphylococcus aureus*, to TB or HIV. In this review we use a rich set of data from studies of sulfadoxine–pyrimethamine resistance in *Plasmodium falciparum* as a framework for considering how to optimize the monitoring and surveillance of new generation antimalarial agents. The goal is to distill lessons that can be applied to other systems, so whenever possible, reviews will be used rather than the primary literature; details can be accessed by consulting the reviews.

2. The definition of antimalarial resistance

Resistance is an operational rather than absolute term. A pathogen is defined as being drug resistant when an infection that normally would have been cured by a treatment regimen survives, multiplies and is transmitted to a new host. In this paradigm a resistant strain is defined with reference to another that is known to be killed by that treatment (i.e. a sensitive strain). This clinical definition is supported, or sometimes supplanted by laboratory studies of the pathogen. For example, assays measuring drug susceptibility *in vitro* can be applied to identify differences between strains. These studies can be complemented by genetic analysis to pinpoint specific mutations that are correlated with the decreased susceptibility to the drug *in vitro* or are more common in the pathogens that survive the standard treatment. In many cases, the reduced susceptibility is not a single major step, but rather a series of modest changes that progressively decrease the parasite responsiveness to treatment. The term "resistance" therefore is always relative, and is used in a wide variety of contexts, the specifics of which are often poorly defined. The confusion created by these different definitions of resistance has important consequences for those who must make decisions about which drugs to recommend for treatment of the disease in question.

3. Measuring clinical outcomes of drug treatment in malaria is complex and expensive

The clinical outcome of drug treatment is central in public health decisions on treatment policy. When a patient is treated, what is the probability that the symptoms will be resolved in a timely way and the infection will be cleared? The clinical assessment of antimalarial efficacy requires an extensive (and expensive) establishment of a clinical team in what is often a poorly resourced endemic setting. This *in vivo* protocol requires enrollment of well-defined patient cohorts with uncomplicated malaria, who are administered supervised treatment with drugs of verified quality and followed up for a set period of time. The standard protocol for assessment of drug efficacy in falciparum malaria has long been established and is regularly reviewed and revised by the WHO (WHO, 2010).

Despite the importance of these standardized estimates, the basic clinical efficacy study masks considerable complexity and can vary markedly in its implementation. Several important factors confound the interpretation of results. Patients are heterogeneous, differing in age, nutritional status, immune function, drug metabolism, genetic susceptibility to infection and many other factors that cannot be tested routinely or controlled for. Because of these variations in baseline host and parasite factors some patients fail to cure their infection even when the parasite is still intrinsically sensitive, whereas other patients can clear an infection even when the parasite exhibits reduced drug susceptibility. The WHO guidelines set out clear inclusion and exclusion criteria to minimize these biases, but in practice, investigators often deviate from these to accommodate variations in the rationale of their study.

Clinical studies also differ markedly in the analytical methods adopted. WHO guidelines allow a number of different methods of data analysis; per protocol, modified intention to treat or survival analysis are all used appropriately in specific situations. However, application of these techniques even to the same data set can introduce differences in derived estimates of drug efficacy that can exceed 30% (Verret et al., 2009). Moreover, the complexity and expense of clinical studies means that the number of patients studied may be constrained. Small sample sizes can result in wide confidence intervals in any derived efficacy estimates. The reality of all of these factors is that 100% clinical cure cannot be expected, even under the best of circumstances, and a variety of other approaches for identifying truly resistant pathogens are needed to provide additional context.

4. Sulfadoxine–pyrimethamine as a paradigm for antimalarial resistance definition

Eukaryotic parasite resistance is exemplified by *P. falciparum*. The rise of antimalarial drug resistance has dominated global malaria control programs since resistance to chloroquine was first documented in patients in 1959. (Contacos et al., 1965; Young and Burgess, 1959). Chloroquine was the first widely used modern antimalarial (Peters, 1987), but within little more than a decade after its introduction chloroquine resistant (CQR) *P. falciparum* had emerged and begun to spread across SE Asia. In areas of high grade CQR a new drug; Fansidar® (sulfadoxine–pyrimethamine, SP) was adopted as the recommended therapy for uncomplicated falciparum malaria. SP was used extensively and gained a reputation as a single dose regimen that was safe, affordable and effective. However by the 1980s its clinical efficacy was clearly waning in SE Asia and alternative treatment regimens had to be deployed (Peters, 1985). Chloroquine retained efficacy for longer in Africa and Latin America and as result, the first line use of SP was only deployed in these regions in the mid 1990s. Everywhere SP was intensively used, the clinical efficacy of SP declined rapidly over a period of a few years (Plowe, 2003; Sibley et al., 2001).

Thus, SP resistance developed and spread "before our very eyes" at a time when many key tools were available to define the mechanism and epidemiology of the resistance, and to identify its clinical and public health consequences (Plowe, 2003).

4.1. Analyzing parasites in the laboratory: parasite genes

The tools of molecular biology had a major impact on our understanding of SP resistance. The target enzymes for both pyrimethamine and sulfadoxine were already known from biochemical studies of related drugs in bacteria and human cells (Hitchings, 1989; Hitchings and Burchall, 1965) A combination of genetics

and *in vitro* analysis of drug susceptibility allowed the genetic changes to be identified in the homologous *P. falciparum* dihydrofolate reductase and dihydropteroate synthase genes that were associated with poor clinical outcomes after SP treatment (Peterson et al., 1988; Triglia and Cowman, 1999). Molecular analysis of parasites could be achieved by polymerase chain reaction (PCR) to amplify the small amount of parasite DNA in a spot of blood from an infected person and simple analytical procedures were designed to identify the relevant genetic changes. Such procedures were relatively inexpensive and accessible, and the methodology to determine these SP-resistance-related genotypes was widely applied in the field (Plowe et al., 1998). The prevalence of particular alleles of the *Pfdhfr* and *Pfdhps* was measured in a wide variety of locations, and patients infected with parasites of particular genotypes were demonstrated to have a significantly higher probability of failing SP treatment (Picot et al., 2009). As SP use increased, concomitant changes were observed in the overall parasite population – a rising prevalence of these resistance – associated alleles in both patients and asymptomatic carriers of *P. falciparum* (Plowe, 2005). The prolonged period over which SP was adopted in various countries in Africa and Latin America provided an opportunity for developing, validating and applying these new tools for tracking antimalarial responses temporally and geographicaly (Ariey et al., 2002; Cortese et al., 2002; Wernsdorfer and Payne, 1991; Wongsrichanalai et al., 2002). These approaches made "molecular epidemiology" of SP resistance a reality, and set the stage for both tracking and modeling the trajectory of the resistance (Hastings and Watkins, 2006; Hastings, 1997).

4.2. Analyzing parasites in the laboratory: drug sensitivity

Acceptance of the molecular indicators of emerging SP resistance was facilitated by studies of parasite responses to sulfadoxine and pyrimethamine in the laboratory. Parasites from patients who had failed SP treatment were adapted to growth in the laboratory, and the correlation between the resistant alleles of *Pfdhfr* and *Pfdhps* and reduced susceptibility to each drug *in vitro* was demonstrated clearly (Basco, 2003; Eldin de Pecoulas et al., 1995; Mberu et al., 2000; Watkins et al., 1985). This approach allowed resistance to each component of SP to be examined individually, and to explore cross resistance to other drugs. Moreover, longitudinal studies highlighted decreasing parasite susceptibility to sulfadoxine and pyrimethamine as the prevalence of the resistant alleles increased in the population (Basco and Ringwald, 1998; Mberu et al., 2000; Menemedengue et al., 2011).

4.3. Pharmacology matters: did the patient have enough drugs?

Treatment failure does not necessarily signify parasite drug resistance. Clinical failure often reflects an inadequate concentration of the drug in the patient's blood (Barnes et al., 2008). These "pharmacological failures" were often overlooked, due to the logistics and cost of pharmacokinetic analysis. Retrospective studies have shown that antimalarial dosing strategies in some vulnerable target populations frequently require adjustment. In the case of SP, dosing was based on strategies derived from pharmacokinetic studies conducted mainly in adults. Appropriate studies conducted many years after SP was first deployed suggested that young children had been systematically under dosed, a factor that almost certainly contributed to the rapid decline in SP efficacy (Barnes et al., 2006, 2008). The relative simplicity of the detection methods (Hitchings et al., 1952) and the very long half-lives of both pyrimethamine and sulfadoxine were important factors in highlighting the crucial role that drug concentrations play in assessment of drug efficacy (Dzinjalamala et al., 2005).

4.4. SP parameters somewhat simplified clinical studies

The pharmacodynamics of sulfadoxine and pyrimethamine afforded an advantage in clinical studies. Both sulfadoxine and pyrimethamine have long half-lives (Bell et al., 2011), providing a significant prophylactic value, and contributing to their ready acceptance both by patients and providers. The fact that a complete treatment of SP required only a single dose also meant that all therapy was directly observed, and this eliminated questions about patient compliance, an issue in many studies.

Molecular approaches have had a significant impact in the formulating the design of clinical studies of SP efficacy. The WHO protocol originally recommended that patients should be followed for 14 days after treatment. The rationale was that a new infection required about 14 days to be manifest clinically, so one could not be sure that parasites that reappeared after 14 days were really derived from the original infection. However, when parasites that recurred in patients failing treatment were examined genetically, the characteristic *Pfdhfr* and *Pfdhps* mutant alleles were observed far more frequently than in the initial parasite sample (Diourte et al., 1999; Doumbo et al., 2000; Mendez et al., 2007). The resistant parasites had been suppressed, but not eliminated, often manifesting after day 14 as late recrudescent infections. With this insight, it became clear that SP was completely curing patients far less frequently than had been assumed from 14 days studies. In response to these insights, the WHO guidelines were updated to require follow up of 28 days (WHO, 2006).

5. Lessons learned from SP

5.1. Integration of clinical and laboratory studies is essential

One of the most important lessons learned from the clinical and laboratory studies of SP, is that their value was realized only fully when data from many studies and different approaches could be integrated. Fig. 1 is a schematic of the interplay and integration of these various approaches, and illustrates the role of each component in the clear definition of resistance.

Recommendations on drug use and procurement require knowledge of the current status of drug efficacy in a country or region. When a focus of resistance to any drug is identified, additional pressing questions are immediately posed: Has resistance arisen anywhere else? Has the focal resistance spread to other areas? If it has spread, how fast and in what directions are the resistant parasites moving? These same questions had been addressed in tracking the origins and spread of antimalarial resistance. Pioneering studies by Clyde (1954), Payne (1987) and Charmot et al. (1991) reviewed in Plowe (2009) had tracked the decline in clinical efficacy of pyrimethamine and chloroquine clinical efficacy, but integration of the new tools into the studies opened up important new opportunities. For example, examination of the molecular signatures of resistant alleles of *Pfdhfr* showed clearly that a single mutation could confer reduced susceptibility to pyrimethamine *in vitro*, and prolong the time to complete clearance of parasites from the patient (Mendez et al., 2002; Plowe, 2009). However, a mutant allele with two additional mutations had a greater association with clinical failure (Kublin et al., 2002). This triple mutant allele did not arise *de novo* in Africa, but had been imported into East Africa from Southeast Asia, spreading by migration throughout the continent, and rising to high levels only as SP use intensified in a particular location (Certain et al., 2008; Maiga et al., 2007; Noranate et al., 2007; Roper et al., 2003, 2004). In contrast, the mutant *Pfdhfr* alleles in Latin America were indigenous, but spread within the continent, mirroring the same migration pattern had been observed with

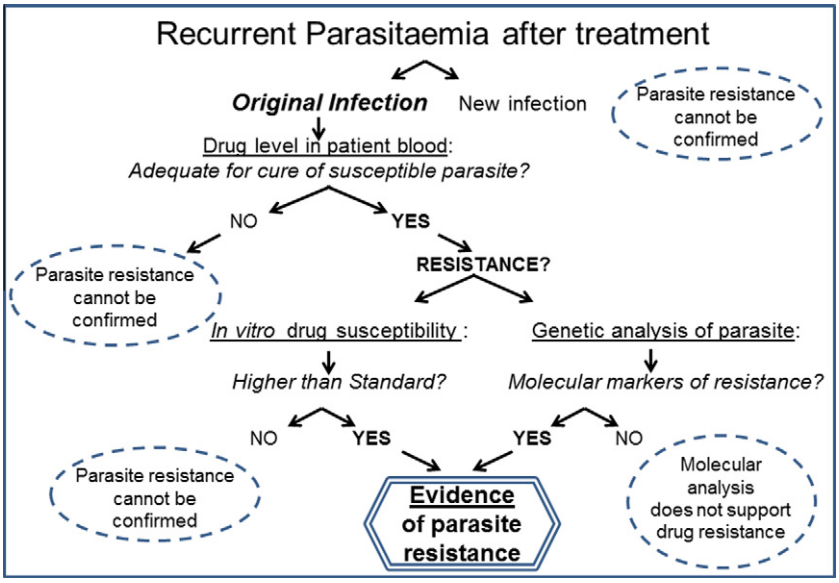

Fig. 1. A simple decision matrix for determining whether a clinical failure is due to parasite drug resistance, or attributable to host or pharmacological factors when a valid molecular marker for that resistance has been identified.

resistance to chloroquine (Ariey et al., 2006; Wootton, 2002), and a more complex history for resistance to the sulfadoxine component of SP (Mita et al., 2011; Pearce et al., 2009).

The combination of these clinical, molecular, pharmacological and *in vitro* analyses allowed more precise definition of resistance to SP, and opened the possibility for lower cost surveillance. A large body of work carried out in many laboratories across the malaria endemic world firmly established the utility of laboratory based approaches as valuable indicators of drug response in the parasite populations, their application complementing clinical data have been used increasingly to inform local policy decisions (Laufer et al., 2006, 2007; Nkhoma et al., 2007).

5.2. Technical challenges: turning data into evidence for public health

Observations of SP resistance clearly demonstrated that clinical failure is often manifest long after molecular and *in vitro* measures of drug efficacy herald the first early warning signs. To harness the power of these surrogate measures their relationships with clinical outcomes needed to be defined. However, attempts to integrate the available supporting information required larger geographic scale analysis of molecular, *in vitro* and pharmacology data to derive metrics that could be applied more widely. Although a plethora of data was available it was scattered among publications, meeting reports and WHO compilations, and in many cases not made public at all. For the most part, only the summary outcomes were available, often long after the studies had been completed. The inherent heterogeneity of data meant that simply comparing the published outputs was not sufficiently informative; inclusion criteria, outcome measures, analytical methods and many other differences precluded pooling of the information directly and its comprehensive analysis.

5.3. Logistic challenges and the growth of networks

By the turn of the millennium it became clear that direct integration of resistance data from the spectrum of scientific approaches and pooling of data from studies over time and geographic location would be required to maximize the impact of the information that had been gathered. Several regional networks were established in the late 1990s both as independent organizations (EANMAT, 2001) and under the auspices of the WHO and the Roll Back Malaria initiative, but a more global approach was needed.

Based on these experiences, a large group of scientists collaborated with the WHO to establish the WorldWide Antimalarial Resistance Network, WWARN (http://www.wwarn.org/). WWARN was initiated as a platform to provide the tools and infrastructure for groups studying all aspects of antimalarial resistance to collaborate easily, and analyze studies productively (Sibley et al., 2008, 2010). It was recognized that these kinds of collaborative analysis could only be accomplished if disparate datasets could be pooled and examined collectively. To accommodate different study designs, data formats and examined variables, the WWARN database has been designed to receive files of individual patient or parasite data in any format, and transform the data sets into a common format, using agreed data structures. Such a repository then allows pooling and standardization, facilitating collaborative analyses of the much larger combined data sets with greater statistical power. With this approach, exploration of correlations between clinical outcomes, patient drug levels, allelic prevalence of potential molecular markers and *in vitro* drug susceptibility become feasible. Individual studies can be incorporated into a global resource and become a far more powerful tool for the surveillance of antimalarial drug efficacy, understanding the underlying biological processes of the parasite, and monitoring temporal and geographic trends in drug resistance.

6. Applying the lessons learned

6.1. Artemisinin combination therapies, ACTs

The early 2000s saw the declining efficacy of a number of drugs that had been used as monotherapies in various regions, including mefloquine, chloroquine and amodiaquine (Lim et al., 2010). New antimalarial agents and therapeutic strategies were needed. A pivotal advance in the last decade has been the deployment of artemisinin combination therapy (ACT). The artemisinins are extremely potent antimalarial agents capable of reducing the parasite biomass by as much as 10^5 within 48 h of starting treatment (White, 2004). The combination of an artemisinin derivative with

a long acting partner drug with a different mode of action, is proposed to reduce the likelihood that a parasite would evolve resistance to both drugs simultaneously (White and Olliaro, 1996).

In Southeast Asia, both chloroquine and SP had lost efficacy by the mid 1980s (Wongsrichanalai et al., 2002), and had been replaced by mefloquine. Resistance to mefloquine evolved in the 1990s, but a series of clinical trials demonstrated that the addition of artesunate to restored efficacy (Nosten et al., 2000; Price et al., 2004), and that this was sustained over more than a decade (Carrara et al., 2009). This approach gained momentum (Breman et al., 2004; Duffy and Mutabingwa, 2004) and in 2006, WHO expert committees advocated strongly for the wide spread deployment of ACTs (WHO, 2006). This strategy is now recommended universally for the treatment of uncomplicated falciparum malaria (WHO, 2010), with almost all endemic countries endorsing their use as first line policy.

6.2. Resistance to ACTs

In the last decade artemisinin combination therapy has become a key component of malaria control and elimination efforts. It was of great concern therefore when reports of declining efficacy of artesunate began to surface from the western region of Cambodia (Lim et al., 2009; Rogers et al., 2009) and from the Thai-Myanmar border (Carrara et al., 2009; Phyo et al., 2012; Cheeseman et al., 2012). The mechanism of resistance to mefloquine had already been identified (Price et al., 1999, 2004) so it was assumed initially reduced efficacy of the mefloquine-artesunate regimen reflected continuing evolution of underlying mefloquine resistance (Alker et al., 2007; Lim et al., 2010). However more detailed clinical studies on a small number of patients in Western Cambodia demonstrated that many of these patients carried parasites with markedly slower response to artemisinin alone (Dondorp et al., 2009, 2010; Noedl et al., 2008). Delayed parasite clearance had been shown previously to be a hallmark of chloroquine, SP and mefloquine resistance (Mendez et al., 2002; Price and Nosten, 2001; ter Kuile et al., 1992). With this in mind, the reduced susceptibility of these parasites to artemisinins was viewed as the first evidence of the development of clinically significant resistance. Since artemisinins are a crucial component of all ACTs, the prospect of losing these drugs to artemisinin resistance was recognized as a major public health threat (White, 2010). The WHO led the effort to coordinate approaches for containment of these parasites, and developed the Global Plan for Artemisinin Resistance Containment, GPARC (WHO, 2011a).

6.3. Better tools for characterizing artemisinin resistance

The surveillance strategy for artemisinin resistance was developed from early observations of the clinical response, despite awareness of the confounding influence of both host and parasite factors. Molecular analyses demonstrated that the genotype of the parasites was an important determinant of slow parasite clearance (Anderson et al., 2010). However, the rate of clearance of parasites in individual patients was idiosyncratic, depending on a range of factors including: the initial parasite biomass, the cell cycle of the particular infection and host immunity. Methods needed to be designed which would allow standardized comparisons between individual patients and between parasite populations (White, 2011). The initial analyses measured the rate of decline of parasites according to the proportion of patients still parasitaemic at various time points after treatment (Dondorp et al., 2009; Noedl et al., 2008). Subsequently a collaborative effort using pooled individual data from various studies allowed the design of a more robust and discriminating approach to standardize using the slope of the parasite clearance response (Flegg et al., 2011). In some patients delayed clearance and late recrudescence following ACTs can be attributed to inadequate drug concentrations (Ashley et al., 2007). In response, pharmacological methods were developed to improve the reliability of detecting artemisinins (Hanpithakpong et al., 2009) and partner drugs (WHO, 2011b) and these measures were added as routine components to prospective studies.

These advances helped to define more precisely the phenotype. They also highlight an inherent conflict between the provision of a robust and sensitive phenotypic assay and the design of a simple and practical field applied tool. For instance the analysis of parasite clearance times requires assessment of the parasite dynamics every 6–8 h during the first 2–3 days after treatment, a step which is a far more labor intensive protocol than current standard efficacy studies. An integrated analysis that allows comprehensive understanding of SP resistance is needed for artemisinin resistance, and development of methods for simple, rapid identification of other sites with similar parasite clinical response are now a high priority.

In the last decade there has been increasing recognition that the analysis of blood concentrations of antimalarial drugs is a critical parameter for defining drug resistance. Full profiling of pharmacokinetic profiles is rarely feasible in clinical trials due to the requirement for intense blood sampling. However sparse sampling at key time points, has proved to be a reliable surrogate for the area under the curve (AUC) for some slowly eliminated drugs; a parameter that correlates with therapeutic failure (White et al., 2008). However, the accurate measurement of artemisinins in blood is technically demanding, and requires sophisticated equipment that is not widely available (Dondorp et al., 2009; Lindegardh et al., 2008). Developing capacity to perform these pharmacological assays is also needed.

Currently, neither genotypic nor *in vitro* assays can distinguish reliably parasite populations that will respond slowly to the artemisinins. Modern approaches for comparing whole genomes are being applied to parasites from the Mekong and other regions with the goal of identifying genetic markers that correlate with the slow clearance phenotype (Campino et al., 2011). Such methods are now being applied much earlier in drug development so that molecular markers of resistance will be available before the novel compounds get to the market (Wu et al., 2011), a clear step forward in detecting and managing resistance to new drugs. Novel approaches for assessing *in vitro* drug susceptibility are underway in several laboratories focused on drug responses, and changes in transcription and metabolic patterns (Davis et al., 2011; Mint Lekweiry et al., 2012; Mok et al., 2011).

6.4. Containing the threat of artemisinin resistance

The major efforts to contain artemisinin resistance are focused on the Mekong region, in the hope of preventing its spread to other regions. Surveillance systems are being established to delineate the spread of the slow responding parasites. Application and integration of all of the methods under development will be needed. The WHO have proposed the Global Plan for Containment of Artemisinin Resistance (WHO, 2011a) in which standardized surveillance strategies will be applied in other regions where similar strains could emerge and quickly spread. Until the surrogate markers are developed, a surveillance matrix for the slow clearance phenotype in regions outside the Mekong will be crucial. A practical surveillance system that includes sites representative of diverse transmission and demographic profiles needs to be mounted, based on a network of teams which can define the baseline of parasite clearance of artemisinin susceptible parasites and regularly monitor and report any changes. Warning signs of slow clearance should trigger a regional or national rapid response team which

can quickly validate or dismiss local reports. The output from such a network must be publically available in real time, and present data in a format that is compatible with the needs of decision makers so that timely appropriate responses can be mobilized.

The scientific community has been able to track resistance to SP, chloroquine and mefloquine retrospectively, but we cannot afford to follow resistance to artemisinins and their partner drugs in the same way. Future effort must be community-wide, intensive and well-coordinated. Study designs need to be creatively planned and performed in disparate areas particularly where little information is currently available. Online provision of the necessary tools, resources and analytical methods will facilitate this process. Sharing the available data will allow subtle trends in changing phenotypic and genotypic trends to be appreciated in as close to real time as possible. These goals lie at the center of the WWARN repository which seeks to provide the platform and tools to facilitate this process.

The task at hand is to adapt tools that have been honed from prior experiences with drug resistance to chloroquine, SP and mefloquine. Complementary work has pioneered developments in information technology and cartography, to define transmission patterns of malaria (Elyazar et al., 2011; Gething et al., 2011a,b), population densities and human migration (Tatem and Smith, 2010). These provide a crucial framework onto which detailed maps can be overlaid depicting drug resistance, malaria prevalence, drug quality and access to antimalarial drugs. This intelligence can then be integrated with the data that have been collected on newer antimalarials, to develop predictive models with which to develop appropriate control and containment strategies (Smith et al., 2010). Only with this concerted, coordinated approach can we gather and analyze the needed information to optimize and apply strategies for containment of resistance to artemisinins in a timely manner.

Conflict of interest

None declared.

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